의학석사 학위논문

Identification of useful immunohistochemical markers for distinguishing thymic squamous cell carcinoma from type B3 thymoma

흉선 편평상피암종과 B3 유형 흉선종의 감별을 위한 유용한 면역조직화학염색 마커의 확인

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지도교수 정 경 천

이 논문을 의학석사 학위논문으로 제출함

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Identification of useful immunohistochemical markers for distinguishing thymic squamous cell carcinoma from type B3 thymoma

by

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ABSTRACT

Introduction: Type B3 thymoma and thymic squamous cell carcinoma (SqCC) often cause a diagnostic problem due to their histological similarities. I aimed to determine whether the expression of 11 markers could distinguish thymic SqCC from type B3 thymoma.

Methods: Immunohistochemical staining was performed on tissue microarray sections from 32 patients, comprising 15 with type B3 thymoma and 17 with thymic SqCC. Staining intensities of CD205, RANK, RANKL, CD40, FGF7, FGFR2, C-kit, and CD5 were graded as low or high expression by manual pathologist-based assessment, and percentages of tumor cell nuclei positive for EZH2, BMI1 and H3K27triMe were scored by automated image analysis to compare expression levels of the markers.

Results: Expression levels of CD205, RANKL, FGFR2, EZH2, BMI1, and C-kit were significantly different between type B3 thymoma and thymic SqCC (*P*-values of <0.001 to 0.045). The area under the receiver-operator characteristic curves (AUC) for the above markers for distinguishing thymic

SqCC from type B3 thymoma ranged from 0.647 to 0.878 (*P*-values of <0.001 to 0.080). The best marker for distinguishing thymic SqCC was EZH2, which was superior to C-kit in terms of sensitivity (94.0 *vs*. 82.4%) and specificity (80.0 *vs*. 66.7%). Furthermore, a combination of C-kit and CD205 with EZH2 modestly improved the diagnostic performance with an AUC of 0.992 (100% sensitivity and 93.3% specificity) for distinguishing thymic SqCC from type B3 thymoma.

Conclusions: A combination of EZH2, C-kit and CD205 can be used as a sensitive and specific marker to distinguish thymic SqCC from type B3 thymoma.

Keywords: EZH2, C-kit, CD205, thymic tumor, immunohistochemistry

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Primary thymic epithelial tumors are rare and have been classified into thymoma and thymic carcinoma. The World Health Organization (WHO) classification of thymic tumors, which was proposed in 1999 and revised in 2004, divides the tumors into types A, AB, B1, B2, B3, and thymic carcinoma, on the basis of morphology and lymphocyte-to-epithelial cell ratio of the tumor, and the degree of cytological atypia of neoplastic epithelial cells.[1] Although the role of this classification with regard to prognosis has not yet been completely validated,[2] a prognosis of type B3 thymoma appears to fall between that of thymic squamous cell carcinoma (SqCC) and other types of thymoma,[3] making the differentiation between type B3 thymoma and thymic SqCC an important role of the pathologist.

Currently, thymic SqCC is differentiated from type B3 thymoma based on both the histological features described above and several immunohistochemical markers, such as C-kit and CD5. [1,4-8] However, differential diagnosis of type B3 thymoma and thymic SqCC histologically is

sometimes difficult due to their morphological similarities,[2,3,9,10] and although C-kit and CD5 have been reported to be useful for distinguishing thymic SqCC, they had limited sensitivities for distinguishing thymic carcinoma.[2-7] Thus, there have been attempts to identify more useful immunohistochemical markers than C-kit and CD5 for distinguishing thymic SqCC from type B3 thymoma.[2,3,10,11]

In this study, I aimed to establish by immunohistochemistry whether the expression of 11 markers, including C-kit and CD5, is significantly different between type B3 thymoma and thymic SqCC and, if they are, the degree to which they can distinguish these conditions. Further, since single markers typically lack the sensitivity and specificity necessary for distinguishing thymic SqCC,[12,13] I determined whether various combinations of the 11 markers were superior to the immunohistochemical results of the individual markers for distinguishing thymic SqCC from type B3 thymoma.

MATERIALS AND METHODS

Patient selection and clinicopathologic evaluation

A total of 32 patients were recruited, including 15 with type B3 thymoma and 17 with thymic SqCC, who underwent surgery between 1997 and 2007 at the Seoul National University Hospital. Tumor tissues were collected from all 32 patients. Hematoxylin and eosin (HE)-stained slides of formalin-fixed, paraffin-embedded surgical specimens were reviewed to determine the histological subtype according to the WHO classification (Fig. 1A and 2A).[1] Other pathologic data, including Masaoka's stage and lymph node status, were also obtained. The clinical data, including age and gender, were obtained from the medical records. This study was approved by the institutional Review Board for human subject research at Seoul National University Hospital (IRB No. C-1301-002-453).

Tissue microarray

Tissue microarray (TMA) blocks (Superbiochips Laboratories, Seoul, Korea)

containing 15 type B3 thymoma and 17 thymic SqCC cases were prepared. Briefly, after a representative tumor area was carefully selected and marked on an HE-stained slide, two core tissue biopsies (2-mm diameter) were taken from the corresponding donor paraffin block and arranged in a recipient paraffin block (TMA block) using a trephine. Cases were considered to represent a tumor if the tumor occupied more than 10% of the core area.

Immunohistochemistry

Formalin-fixed, paraffin-embedded TMA blocks were sectioned into 4-mm slices and affixed onto glass slides. Sections were subjected to immunostaining with 11 antibodies using BenchMark XT (Ventana, Tucson, AZ, USA), BOND-MAX (Leica Microsystems, Bannockburn, IL, USA) or autostainer 360 (Lab Vision, Fremont, CA, USA) systems, according to the manufacturer's instructions (Table 1). For CD205, RANK, RANKL, CD40, FGF7, FGFR2, C-kit, and CD5, the staining intensity of the cytoplasm or membrane was recorded as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong) by manual pathologist-based assessment, and categorized as low or

high expression. The thresholds between low and high expression were set for each marker individually (Tables 3-4). For EZH2, BMI1 and H3K27triMe, digital images were captured using the ScanScope CS slide scanner (Aperio Technologies, Vista, CA, USA) and analyzed using the ImageScope software and the Nuclear v9 algorithm (version 11.2.0.782, Aperio Technologies, Vista, CA, USA). The percentage of tumor cell nuclei positive in each case was calculated in one representative area at a magnification of ×200.

Table 1. Primary antibodies used in the immunohistochemical staining

Antibody	Company	Clone	Ag retrieval	Dilution	Detection method
Thymic epithelial development-related markers					
CD205	Novocastra	11A10	Heat for 30 min	1:50	Ultra view polymer
RANK	R&D Systems	80707	Heat for 30 min	1:50	Ultra view polymer
RANKL	R&D Systems	70525	Heat for 30 min	1:40	Ultra view polymer
CD40	Santa Cruz	LOB-11	Heat for 20 min	1:30	Bond polymer kit
FGF and FGFR families					
FGF7	Santa Cruz	Goat polyclonal	Heat for 20 min	1:100	Vectastain Elite ABC kit
FGFR2	Santa Cruz	Rabbit polyclonal	Heat for 30 min	1:200	Ultra view polymer
Polycomb group proteins and H3K27triMe					
EZH2	Novocastra	6A10	Heat for 20 min	1:200	Bond polymer kit
BMI1	Upstate	F6	Heat for 20 min	1:200	Bond polymer kit
H3K27triMe	Abcam	6002	Heat for 20 min	1:100	Bond polymer kit
Routinely used markers					
C-kit	Dako	Rabbit polyclonal	Heat for 30 min	1:100	Ultra view polymer
CD5	Novocastra	4C7	Heat for 60 min	1:100	Ultra view polymer

Statistical analysis

Continuous variables were analyzed using Student's *t*-test or the Mann–Whitney test, and categorical variables were analyzed using the χ^2 test. Pearson's correlation test or Spearman's rank-correlation test was used to identify associations between age and the various immunohistochemical markers. A logistic regression model was used to combine the immunohistochemical markers, and receiver-operator characteristic (ROC) curves were constructed to calculate the area under the ROC curve (AUCs) for markers and to estimate their sensitivity and specificity. AUCs were compared to determine which marker(s) resulted in the best diagnostic performance. Detailed descriptions of logistic regression models and ROC curve analyses are given below. Correction for multiple comparison testing was not used in these exploratory analyses. All *P*-values that were two-sided and less than 0.05 were considered to indicate statistical significance, and values between 0.05 and 0.10 were considered to indicate marginal significance. All statistical analyses were performed using the SPSS for Windows (release 15.0.0, SPSS Inc., Chicago, IL, USA), Prism for Windows

(version 4.02, GraphPad Software, La Jolla, CA, USA) or MedCalc for Windows (version 12.3.0.0, MedCalc Software, Mariakerke, Belgium).

Logistic regression analysis

A logistic regression model was used to generate a function that combined the immunohistochemical results of several markers. Predicted probabilities were calculated for each case from that function and served as scores of a 'combined marker' for construction of a ROC curve.[13] Although Box–Tidwell transformation confirmed that continuous markers and their interaction terms did not violate the linear assumption of the logit,[14] the data were transformed to the natural logarithmic scale, which provided a better fit with a higher log-likelihood value. Since the goal was simply to predict probabilities as accurately as possible from these data, multicollinearity was not assessed among the continuous markers and interaction terms. Thus, the function was generated from the logistic regression model using the categorical markers, natural logarithms of the continuous markers and interaction terms.

ROC curve analysis

ROC curves were constructed for the individual and combined immunohistochemical markers. AUCs and the 95% confidence intervals (CIs) were calculated as an overall index of the diagnostic performance of the markers. The AUCs of the individual markers were compared with the chance diagonal of 0.5, and the AUCs of the individual and combined markers were compared on a pair-wise basis using the nonparametric methodology of DeLong *et al.*[15] to test for statistically significant differences in the diagnostic performance between markers. ROC curves also provided estimates of sensitivity and specificity at the threshold to maximize 'sensitivity + specificity - 1' (threshold corresponding to the *Youden index*).[16] The *Youden index* has received much attention in the literature,[17] and Le[18] proposed it as a solution to the problem of determining the optimum threshold for a single classifier within the framework of the ROC curve.

RESULTS

Patient characteristics

The demographic features according to study group are presented in Table 2. There were no differences with respect to mean age or gender between patients with type B3 thymoma and thymic SqCC ($P = 0.552$ and $P = 0.688$, respectively). To determine whether age correlates with staining intensity or the percentage of tumor cell nuclei positive for various immunohistochemical markers in type B3 thymoma or thymic SqCC, the Mann–Whitney test or Pearson's correlation test was performed. These revealed no significant correlation between age and the various immunohistochemical markers (Table 3); thus, age adjustment was not performed for any of the subsequent ROC curve analyses.

Table 2. Patient characteristics

Variables	Type B3 thymoma (n = 15)	Thymic SqCC (n = 17)	*P*-value
Age (years)*	54.93 ± 14.86	58.12 ± 15.04	0.552
Gender†			0.688
Male	9 (60.0%)	9 (52.9%)	
Female	6 (40.0%)	8 (47.1%)	

SqCC, squamous cell carcinoma.

*Data are means ± SDs of age.

†Data are the number of patients.

Table 3. Associations between age and individual markers in patients with type B3 thymoma or thymic squamous cell carcinoma (SqCC)

Variables	Type B3 thymoma (n = 15)*	*P*-value	Thymic SqCC (n = 17)*	*P*-value
CD205		0.679		0.509
Low (≤1+)	58.80 ± 12.95		57.14 ± 16.16	
High (>1+)	53.00 ± 16.01		62.67 ± 8.622	
RANK		0.800		0.703
Low (≤1+)	56.00 ± 9.899		61.00 ± 7.874	
High (>1+)	54.77 ± 15.79		57.23 ± 16.80	
RANKL		…†		0.956
Low (≤2+)	54.93 ± 14.86		59.38 ± 10.31	
High (>2+)	…†		57.58 ± 15.05	
CD40		0.171		0.279
Low (≤0)	52.92 ± 14.90		60.08 ± 16.38	
High (>0)	68.00 ± 56.57		53.40 ± 11.28	
FGF7		0.536		0.432
Low (≤1+)	57.86 ± 13.80		54.67 ± 5.686	
High (>1+)	52.38 ± 16.20		58.86 ± 16.43	
FGFR2		0.859		0.799
Low (≤2+)	56.10 ± 13.31		59.80 ± 8.468	
High (>2+)	52.60 ± 19.07		57.42 ± 17.35	
EZH2	- 0.485‡	0.067	- 0.061‡	0.817
BMI1	0.253‡	0.363	0.227‡	0.380
H3K27triMe	- 0.493‡	0.062	- 0.051‡	0.845
C-kit		0.529		0.824
Low (≤2+)	52.00 ± 17.01		61.00	
High (>2+)	59.33 ± 10.80		57.94 ± 15.51	
CD5		0.536		0.417
Low (≤2+)	53.33 ± 15.01		59.50 ± 16.87	
High (>2+)	61.33 ± 15.18		56.14 ± 12.97	

*Data are means ± SDs of age except where noted.

†Could not be calculated.

‡Pearson's r's.

Comparison of immunohistochemical results between type B3 thymoma and thymic SqCC

CD205 (DEC-205) is a member of the macrophage mannose receptor family of C-type lectins, and is expressed predominantly by the thymic cortical epithelium and dendritic cells.[19] In these cells, CD205 is believed to function as an antigen uptake receptor for presentation via MHC class II molecules.[20] Receptor activator of NF-κB (RANK) is a type I membrane protein that shares high homology with CD40.[21] During thymic development, the cytokine RANK ligand (RANKL), produced by $CD4^{+}CD3^{-}$ lymphoid tissue inducer (LTi) cells, activates RANK signaling, which is essential for medullary thymic epithelial cell development during embryogenesis.[22-24] In addition, CD40 cooperates with RANK to promote medullary thymic epithelial cell development. Based on these results, tumor samples were stained with antibodies against CD205, RANK, RANKL, and CD40 to compare the expression levels of these proteins between type B3 thymoma and thymic SqCC. Expression levels were recorded and categorized as described above. With respect to RANK and CD40, the majority of cases showed strong and

diffuse expression of RANK in a membranous pattern and no expression of CD40 (Fig. 1B-1E, Fig. 2B-2E and Table 4). The expression of RANKL was also low in both type B3 thymoma and thymic SqCC, except for four cases of thymic SqCC. However, the expression level of CD205 was markedly different between type B3 thymoma and thymic SqCC ($P = 0.005$); strong and diffuse expression was observed in type B3 thymoma, and weak expression in thymic SqCC. These results may reflect the immaturity of thymic SqCC cells.

Keratinocyte growth factor/fibroblast growth factor 7 (KGF/FGF7) belongs to the FGF family and, along with FGF10, is a distinct member in that it has a stromal origin but appears to act specifically on epithelial cells.[25-27] FGFR2-IIIb, a receptor for FGF7 and FGF10 that is expressed in thymic epithelial cells, has been reported to play an important role in the development and reconstitution of the thymus.[28-30] To investigate whether these proteins are expressed in type B3 thymoma and thymic SqCC, tumor samples were stained with antibodies against FGF7 and FGFR2, and the expression levels were recorded and categorized as described above. The FGF7 expression level tended to be higher in thymic SqCC, but the difference was only marginally

significant (P = 0.077; Fig. 1F-1G, Fig. 2F-2G and Table 4). In contrast, cytoplasmic FGFR2 expression was significantly higher in thymic SqCC than in type B3 thymoma (P = 0.035).

EZH2, as a form of polycomb repressive complex 2 (PRC2), induces silencing of hundreds of genes via trimethylation of lysine 27 in histone H3 (H3K27triMe) through cooperation with BMI1, the core subunit of polycomb repressive complex 1.[31-34] EZH2 first requires deacetylation by endogenous histone deacetylase (HDAC) to trimethylate H3K27,[35-37] and a recent study suggested that HDAC may correlate with the aggressiveness of thymic carcinoma.[38] Based on these data, tumor samples were stained with antibodies against EZH2, BMI1 and H3K27triMe to determine the percentages of tumor cell nuclei positive for these proteins in type B3 thymoma and thymic SqCC. While the percentage of tumor cell nuclei positive for EZH2 was significantly higher in thymic SqCC (P < 0.001; Fig. 1H-1J, Fig. 2H-2J, Table 4 and Fig. 3), that of BMI1 was markedly lower in thymic SqCC than in type B3 thymoma (P = 0.033). The percentage of nuclei positive for H3K27triMe varied among tumors (P = 0.176).

The expression level of CD5 in type B3 thymoma and thymic SqCC was not different (P = 0.197), but that of C-kit was markedly higher in thymic SqCC than in type B3 thymoma (P = 0.001), demonstrating weak expression in type B3 thymoma and strong and diffuse expression in thymic SqCC (Fig. 1K-1L, Fig. 2K-2L and Table 4).

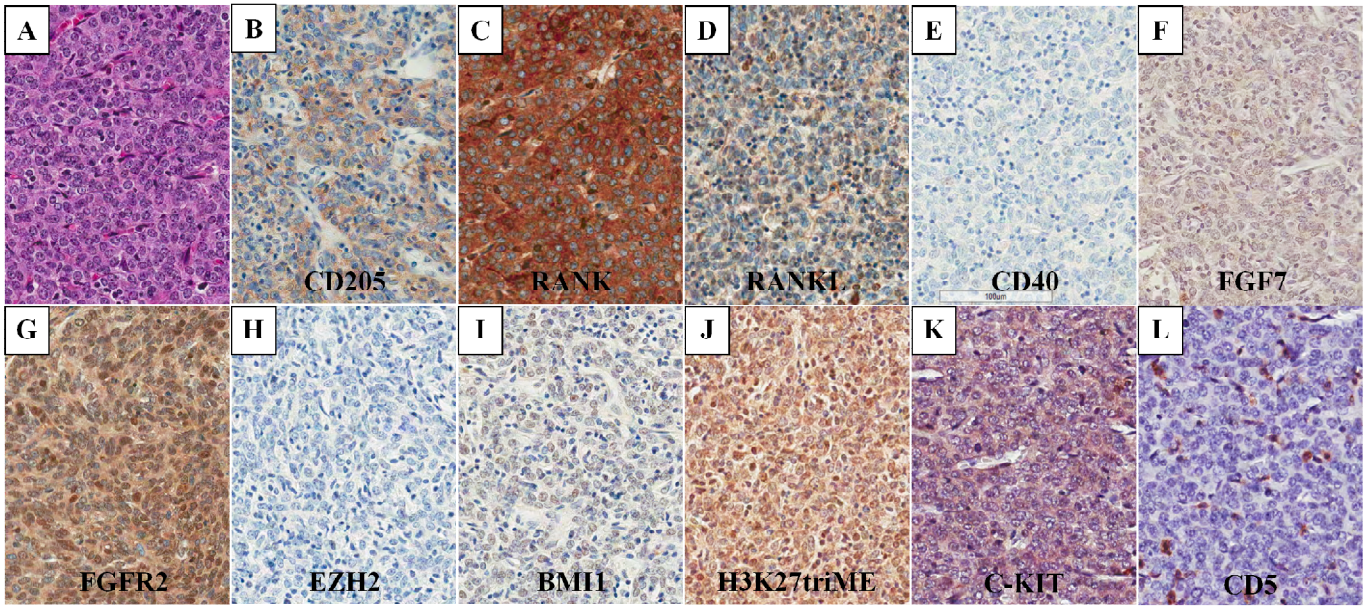


Figure 1. Histological features and an immunohistochemical profile of type B3 thymoma. (A) Polygonal, medium-sized tumor cells were arranged in solid sheets. The nuclei were round or elongated. Strong expression of (B) CD205 and (C) RANKL in a cytoplasmic staining pattern, and (G) BMI1 in a nuclear staining pattern with weak or absent expression of (D) FGF7, (E) FGFR2, (F) EZH2, and (H) C-kit was a typical profile of type B3 thymoma (A-H: magnification, ×200).

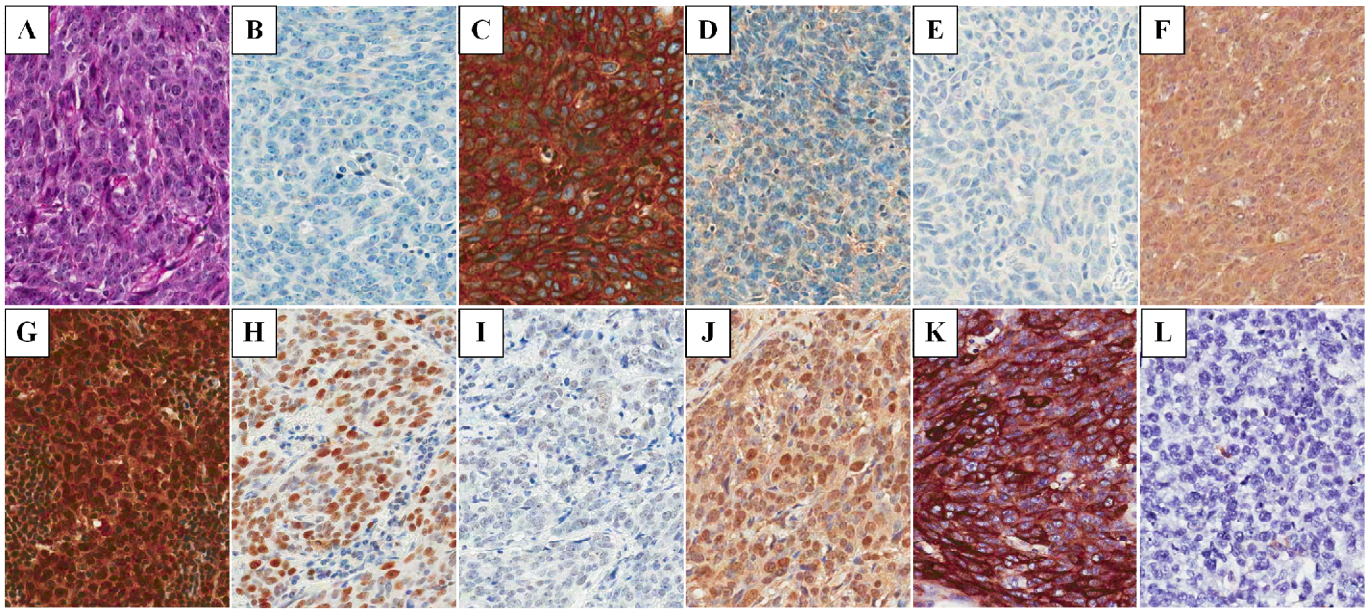


Figure 2. Histological features and an immunohistochemical profile of thymic squamous cell carcinoma (SqCC). (A) Large, atypical tumor cells were arranged in nests or cords. Loss of expression of (B) CD205 and (G) BMI1 with strong expression of (C) RANKL, (D) FGF7 and (E) FGFR2 in a cytoplasmic staining pattern, (F) EZH2 in a nuclear staining pattern, and (H) C-kit in a membranous pattern was a typical profile of thymic SqCC (A-H: magnification, ×200).

Table 4. Immunohistochemistry results

Variables	Type B3 thymoma (n = 15)*	Thymic SqCC (n = 17)*	*P*-value
CD205			0.005
Low (≤1+)	5 (33.3%)	14 (82.4%)	
High (>1+)	10 (66.7%)	3 (17.6%)	
RANK			0.461
Low (≤1+)	2 (13.3%)	4 (23.5%)	
High (>1+)	13 (86.7%)	13 (76.5%)	
RANKL			0.045
Low (≤2+)	15 (100%)	13 (76.5%)	
High (>2+)	0 (0%)	4 (23.5%)	
CD40			0.272
Low (≤0)	13 (86.7%)	12 (70.6%)	
High (>0)	2 (13.3%)	5 (29.4%)	
FGF7			0.077
Low (≤1+)	7 (46.7%)	3 (17.6%)	
High (>1+)	8 (53.3%)	14 (82.4%)	
FGFR2			0.035
Low (≤2+)	10 (66.7%)	5 (29.4%)	
High (>2+)	5 (33.3%)	12 (70.6%)	
EZH2	6.462 ± 12.73†	27.72 ± 22.76†	<0.001
BMI1	43.29 ± 24.62†	24.74 ± 24.33†	0.033
H3K27triMe	22.14 ± 10.15†	30.67 ± 17.84†	0.176
C-kit			0.001
Low (≤2+)	12 (80.0%)	4 (23.5%)	
High (>2+)	3 (20.0%)	13 (76.5%)	
CD5			0.197
Low (≤2+)	12 (80.0%)	10 (58.8%)	
High (>2+)	3 (20.0%)	7 (41.2%)	

SqCC, squamous cell carcinoma.

*Data are the number of patients except where noted.

†Means ± SDs of the percentage of positive tumor cell nuclei.

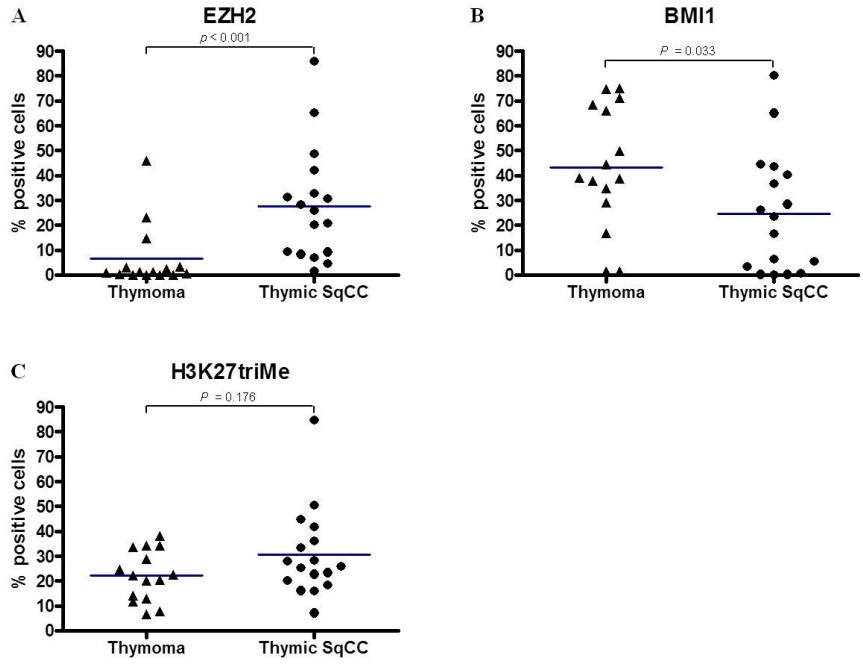


Figure 3. Scatter plots showing the percentages of tumor cell nuclei positive for (A) EZH2, (B) BMI1, (C) H3K27triMe in patients with type B3 thymoma or thymic squamous cell carcinoma (SqCC). Those were assessed by the Aperio ScanScope slide scanner, the ImageScope software, and the Nuclear v9 algorithm as described in Materials & Methods. The percentages of tumor cell nuclei positive for EZH2 and BMI1, but not for H3K27triMe, were significantly different between type B3 thymoma and thymic SqCC. The horizontal lines denote the mean percentages.

Utility of individual immunohistochemical markers for distinguishing thymic SqCC

To more accurately evaluate the diagnostic values of the selected markers, ROC curves were constructed and AUCs of the individual markers were calculated. CD205, RANKL, FGF7, FGFR2, EZH2, BMI1, and C-kit, which showed significant or marginally significant differences in expression levels between type B thymoma and thymic SqCC, were first selected. In addition, although CD5 expression was not unique to thymic SqCC, the ROC curve for CD5 was also constructed since it, together with C-kit, is routinely used to distinguish thymic carcinoma from thymoma. Next, their AUCs were compared to one another. The AUCs for CD205, RANKL, FGF7, FGFR2, EZH2, and BMI1 for distinguishing thymic SqCC from type B3 thymoma ranged from 0.647 to 0.878 (*P*-values of <0.001 to 0.080; Table 5). In contrast, the AUCs for C-kit and CD5, both of which are used routinely, were 0.837 and 0.612, respectively ($P < 0.001$ and $P = 0.232$, respectively). The rank order of significant AUCs was EZH2 > C-kit > CD205 > BMI1 > FGFR2. Thus, the single best marker for distinguishing thymic SqCC was EZH2, with

the highest AUC (0.878), sensitivity (94.1%) and specificity (80.0%), followed by C-kit, CD205, BMI1, and FGFR2, in order of their AUCs.

Table 5. AUCs, sensitivities and specificities for selected or routinely used markers for distinguishing thymic squamous cell carcinoma from type B3 thymoma

Markers	AUCs	Sensitivity	Specificity	Threshold	*P*-value*
Selected markers†					
CD205	0.753	82.4%	66.7%	≤1+	0.004
RANKL	0.647	23.5%	100%	>2+	0.080
FGF7	0.675	82.4%	46.7%	>1+	0.054
FGFR2	0.706	70.6%	66.7%	>2+	0.014
EZH2	0.878	94.1%	80.0%	>3.3%	<0.001
BMI1	0.722	64.7%	80.0%	≤28.4%	0.018
Routinely used markers					
C-kit	0.837	76.5%	80.0%	>2+	<0.001
CD5	0.612	41.2%	80.0%	>2+	0.232

*The AUCs of some individual markers were compared with the chance diagonal of 0.5 on a pair-wise basis using the nonparametric methodology of DeLong *et al*.[15] to calculate two-sided *P*-values.

†Only markers that were significant or marginally significant in Table 4 and Figure 3 were selected for these analyses (see text for details).

Overall diagnostic performance of the combined markers

To enhance the overall diagnostic performance of the tests using immunohistochemical markers for distinguishing thymic SqCC, I first combined the percentages of tumor cell nuclei positive for EZH2 and staining intensities of C-kit using a logistic regression model. This combination (EZH2 and C-kit) was preferred because its components showed the highest and second-highest AUCs of 0.878 and 0.837, respectively, in the ROC curve analysis. The ROC curve for the combined marker yielded an AUC of 0.965 (94.1% sensitivity, 93.3% specificity; threshold = 0.4036). This AUC was significantly higher than that of C-kit ($P = 0.030$), but not of EZH2 ($P = 0.171$; Fig. 4A and Table 6).

Next, to make the AUC for the combined marker significantly higher than that of EZH2 for discriminating thymic SqCC from type B3 thymoma, the staining intensity of CD205, the marker with the third-highest AUC (0.753), was added to the logistic regression model. The ROC curve for this combined marker yielded an AUC of 0.992 (100% sensitivity, 93.3% specificity; threshold = 0), which was significantly higher than that of C-kit

and CD205 (P = 0.021 and P = 0.007, respectively), and marginally significantly higher than that of EZH2 (P = 0.081; Fig. 4B and Table 6).

Next, addition of the immunohistochemical results of BMI1 and FGFR2, the markers with the fourth- and fifth-highest AUCs (0.722 and 0.706), to the logistic regression model failed to further increase the AUC of the combined markers (Fig. 4C and Table 6); thus, BMI1 and FGFR2 could be excluded from the logistic regression model.

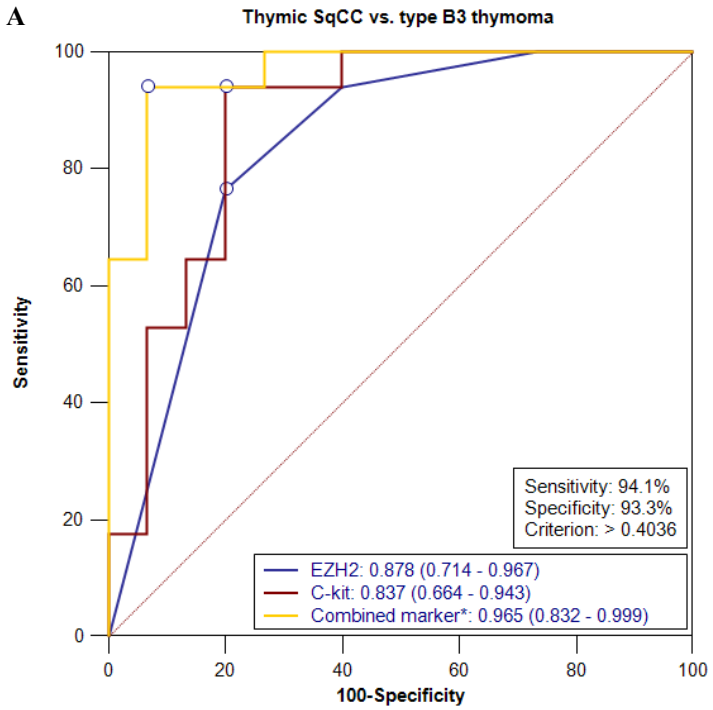
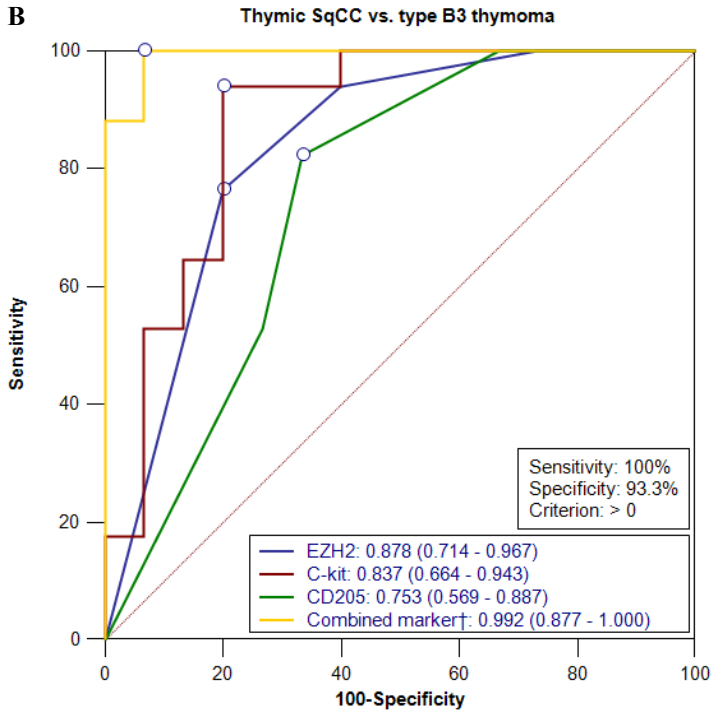


Figure 4. ROC curves for individual markers and their combined marker. ROC curves for (A) EZH2, C-kit, and their combined marker, (B) EZH2, C-kit, CD205, and their combined marker, and (C) EZH2, C-kit, CD205, BMI1, FGFR2, and their combined marker are constructed to discriminate thymic squamous cell carcinoma (SqCC) from type B3 thymoma. The blue-colored numbers on the lower side are AUCs and the 95% confidence intervals. Open circles on each ROC curve denote the points with the *Youden index* respectively. The sensitivity and specificity at the threshold corresponding to the *Youden index* for the combined markers are provided on the lower right-hand side. (Figure continued on next page.)



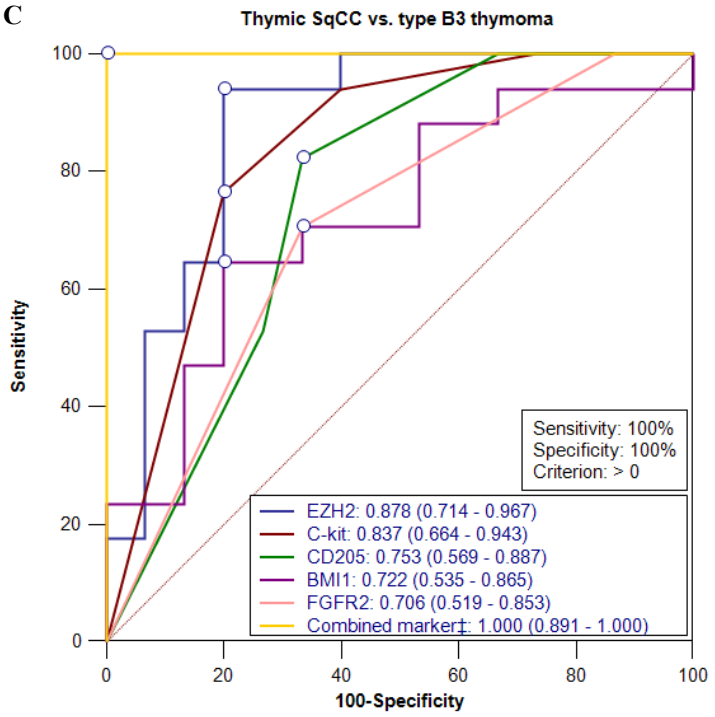


Figure 4. Continued.

Table 6. Pair-wise comparisons of AUCs between individual and combined markers

Markers	Difference between AUCs (95% confidence interval)	*P*-value*
Comparisons of AUCs between EZH, C-kit and their combined marker		
EZH2 *vs*. C-kit	0.041 (-0.142 to 0.224)	0.659
EZH2 *vs*. Combined marker	0.086 (-0.037 to 0.210)	0.171
C-kit *vs*. Combined marker	0.127 (0.013 to 0.242)	0.030
Comparisons of AUCs between EZH, C-kit, CD205, and their combined marker		
EZH2 *vs*. C-kit	0.041 (-0.142 to 0.224)	0.659
EZH2 *vs*. CD205	0.125 (-0.110 to 0.361)	0.297
C-kit *vs*. CD205	0.084 (-0.061 to 0.230)	0.257
EZH2 *vs*. Combined marker	0.114 (-0.014 to 0.242)	0.081
C-kit *vs*. Combined marker	0.155 (0.023 to 0.287)	0.021
CD205 *vs*. Combined marker	0.239 (0.067 to 0.412)	0.007
Comparisons of AUCs between EZH, C-kit, CD205, BMI1, FGFR2, and their combined marker†		
EZH2 *vs*. Combined marker	0.122 (-0.010 to 0.253)	0.070
C-kit *vs*. Combined marker	0.163 (0.026 to 0.300)	0.020
CD205 *vs*. Combined marker	0.247 (0.074 to 0.420)	0.005
BMI1 *vs*. Combined marker	0.278 (0.095 to 0.461)	0.003
FGFR2 *vs*. Combined marker	0.294 (0.130 to 0.459)	0.001

*The AUCs were compared using the nonparametric methodology of DeLong *et al*.[15] to calculate two-sided *P*-values.

†AUCs were compared only between the individual markers *vs*. their combined marker.

Clinicopathological features associated with marker status in patients with thymic SqCC

Lastly, the association between EZH2, C-kit and CD205, which were used to generate a combined marker and the clinicopathological features of thymic SqCC, was investigated. With the exception of a marginally significant association between percentages of tumor cell nuclei positive for EZH2 and gender ($P = 0.059$), no other significant associations were found among EZH2, C-kit and CD205 and various clinicopathological features, including age, gender, Masaoka's stage, and lymph node status in patients with thymic SqCC (P-values of 0.272 to 0.870; Table 7). The combined marker generated from immunohistochemical results of EZH2, C-kit and CD205 was not significantly associated with any of the clinicopathological features listed above in patients with thymic SqCC ($P = 0.495$, 0.236, 0.879, and 0.456, respectively; Table 8).

Table 7. Associations between EZH2, C-kit, and CD205 and clinicopathological features in patients with thymic squamous cell carcinoma

Variables	EZH2*	*P*-value	C-kit†		*P*-value	CD205†		*P*-value
			Low (≤2+)	High (3+)		Low (≤1+)	High (>2+)	
Age	- 0.061‡	0.817	- 0.182§		0.484	0.116§		0.657
Gender		0.059			0.274			0.600
Male (n = 9)	37.56 ± 23.87		0	9		7	2	
Female (n = 8)	16.65 ± 16.37		1	7		7	1	
Stage		0.646			0.506			0.870
Stage I (n = 5)	33.99 ± 29.87		0	5		4	1	
Stage II - IV (n = 12)	25.11 ± 20.09		1	11		10	2	
LN metastasis		0.272			0.398			0.756
No (n = 9)	18.01 ± 12.71		1	8		8	1	
Yes (n = 6)	30.21 ± 22.49		0	6		5	1	

*Data are means ± SDs of the percentage of positive tumor cell nuclei except where noted.

†Data are the number of patients except where noted.

‡Pearson's r's.

§Spearman's ρ's.

Table 8. Associations between the combined marker and clinicopathological features in patients with thymic squamous cell carcinoma

Variables	Combined marker*, †	*P*-value
Age	- 0.178‡	0.495
Gender		0.236
Male (n = 9)	0.992 ± 0.024	
Female (n = 8)	0.911 ± 0.125	
Stage		0.879
Stage I (n = 5)	0.947 ± 0.118	
Stage II - IV (n = 12)	0.957 ± 0.088	
LN metastasis		0.456
No (n = 9)	0.980 ± 0.060	
Yes (n = 6)	0.912 ± 0.137	

*Generated using a logistic regression model to combine the expression levels of EZH2, C-kit and CD205 determined by immunohistochemistry.

†Data are means ± SDs of scores of the combined marker except where noted.

‡Pearson's r's.

DISCUSSION

In this study, immunohistochemical results of CD205, RANKL, FGFR2, EZH2, BMI1, and C-kit differed significantly between type B3 thymoma and thymic SqCC. EZH2 was determined to be the single best marker for distinguishing thymic SqCC, followed by C-kit > CD205 > BMI1 > FGFR2 in order of AUCs. Furthermore, I demonstrated that the combination of EZH2, C-kit and CD205 could discriminate thymic SqCC from type B3 thymoma better than any of the individual markers. The AUC of this combined marker was significantly higher than those of C-kit and CD5, the two markers which were reported to be useful for distinguishing thymic carcinoma,[4-8] and are currently widely used in practice. These findings indicate that the combination of EZH2, C-kit and CD205 can be used as an effective differential diagnostic marker for distinguishing thymic SqCC from type B3 thymoma.

EZH2 is the catalytic subunit of PRC2, which is involved in modifying chromatin at least in part by inducing trimethylation of H3K27, and eventually silencing hundreds of genes.[33,34] With regard to cancer, Varambally

et al.[39] first reported that EZH2 is overexpressed in metastatic prostate cancer and that clinically localized prostate cancers that express high concentrations of EZH2 show a poorer prognosis. They also reported that a reduction in the level of EZH2 protein present in prostate cells inhibits their proliferation *in vitro*.[39] Afterward, there have been many reports of the relationship between overexpression of EZH2 and carcinogenesis, aggressiveness, invasive potentials, and/or a poor prognosis of carcinoma of various origins, including lung, stomach, liver, colon, and urinary bladder.[40-44] EZH2 has also been shown to be useful in the diagnosis of precancerous lesions of the breast and hepatocellular carcinoma of the liver.[45,46] However, whether EZH2 is a useful marker in the diagnosis of thymic carcinoma is unknown. To my knowledge, this is the first report that EZH2 expression determined by immunohistochemistry can be used as a diagnostic tool for thymic SqCC.

A recent phase II trial showed that Belinostat, a pan-HDAC inhibitor, possesses modest antitumor activity in the group of heavily pretreated thymic malignancies,[38] suggesting that HDAC correlates with the aggressiveness of thymic carcinoma. There have been some reports of physical and functional

links between EZH2 and HDAC.[35-37] Methylation of H3K27 by EZH2 is believed to first require endogenous HDAC-mediated deacetylation.[35-37] Thus, although there is no direct evidence that EZH2 expression correlates with tumorigenesis and/or aggressive behavior of thymic carcinoma, EZH2 may also have a pathophysiologic and/or prognostic role in thymic carcinoma, allowing for the close links between EZH2 and HDAC.

C-kit and CD5 have been reported to be useful in the diagnosis of thymic carcinoma.[4-7] C-kit was reported to be positive in 65–91% of thymic carcinomas, as opposed to 0–5% of thymomas.[4,5,47] This is in agreement with my study, which showed positive immunoreactivity for C-kit in 76.5% of thymic SqCC and 20.0% of type B3 thymoma with a high AUC (0.837); thus, I confirmed the usefulness of C-kit as a marker for the diagnosis of thymic SqCC. CD5 was reported to be positive in 50–100% of thymic carcinomas but rare in type B3 thymoma.[4,10,48,49] However, in my study, CD5 was positive in only 41.2% of thymic SqCC cases and 20.0% of type B3 thymoma cases with an AUC of 0.612; thus, CD5 is not likely a useful marker for differentiation of type B3 thymoma and thymic SqCC. This could be explained by the smaller

tissue of TMA and/or the use of an anti-CD5 antibody derived from a different clone. CD205, of which the diagnostic utility in thymic carcinoma was reported to be conflicting,[3,10] was positive in 82.4% of thymic SqCC cases and 33.3% of type B3 thymoma cases, with an AUC of 0.753.

Pepe[13] reported that while no single biomarker is sufficiently sensitive and specific for the differentiation of cancer, a combination of several biomarkers may provide a better diagnostic tool than any single test alone. Thus, a number of the immunohistochemical markers were combined to enhance their diagnostic capability. This combination of markers significantly improved the overall diagnostic performance in terms of distinguishing thymic SqCC from type B3 thymoma, compared to use of single immunohistochemical markers. A logistic regression model was used as a statistical approach to combining the markers. Alternative approaches, such as a modified logistic regression model or an AUC-based objective function model, may improve the numerical strength of the data;[50,51] however, these approaches were not used because a logistic regression model was enough to indicate that the AUC of the combined markers was better than that of any

single marker individually.

The combination of the EZH2, C-kit and CD205 markers resulted in a very high AUC of 0.992 (100% sensitivity and 93.3% specificity), significantly higher than that of C-kit or CD205, but only marginally significantly higher than that of EZH2. This is likely due to the small sample size of this study. In accordance with this speculation, while addition of BMI1 and FGFR2 with the fourth- and fifth-highest AUCs to the existing logistic regression model generated from EZH2, C-kit and CD205 yielded an AUC of 1.000, the latter value was only marginally significantly higher than that of EZH2 alone. This finding suggests that a prospective study of a larger number of cases is required to demonstrate that the marker combination is significantly superior to EZH2 alone for distinguishing thymic SqCC from type B3 thymoma.

Few reports have addressed whether there is a significant association between the expression of EZH2, C-kit and CD205 by immunohistochemistry and clinicopathological features, such as age, sex, staging, and lymph node status. In this study, with the exception of a marginally significant association

between the percentage of tumor cell nuclei positive for EZH2 and gender, there were no significant associations between either the individual or combined markers and the various clinicopathological features. This suggests that either the individual or combined markers could be used to discriminate thymic SqCC of all stages from type B3 thymoma.

In conclusion, my data clearly indicate that the expression of CD205, RANKL, FGF7, FGFR2, EZH2, BMI1, and C-kit by immunohistochemistry differs significantly between type B3 thymoma and thymic SqCC, and that EZH2 could be used as an improved, effective differential diagnostic tool for distinguishing thymic SqCC. However, whether the combination of EZH2, C-kit and CD205 is significantly superior to the single marker, EZH2, for distinguishing thymic SqCC remains unclear. Moreover, how EZH2 contributes to the pathophysiology or prognosis of thymic SqCC remains to be determined. Further researches, including a prospective study that includes a larger number of cases, are required to confirm the utility of the combined marker for distinguishing thymic SqCC and to investigate the role of EZH2 in the pathophysiology of thymic SqCC.

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국문 초록

서론: B3 유형 흉선종과 흉선 편평상피암종은 조직학적 유사성으로 인해 간혹 감별 진단에 어려움이 발생하게 된다. 본 연구에서는 11 개의 마커를 선택하여 이 마커들이 B3 유형 흉선종과 흉선 편평상피암종의 감별 진단에 도움이 되는지 확인하고자 하였다.

방법: 15 명의 B3 유형 흉선종 환자 및 17 명의 흉선 편평상피암종 환자 등 총 32 명의 환자들로부터 얻은 조직 마이크로어레이를 대상으로 면역조직화학염색을 시행하였다. 병리 의사의 판독을 통해 CD205, RANK, RANKL, CD40, FGF7, FGFR2, c-Kit, 및 CD5 의 발현 수준을 낮은 수준과 높은 수준으로 구분하였으며, 자동화된 이미지 분석 방법을 통해 EZH2, BMI1 및 H3K27triMe 가 양성인 암세포의 백분율을 계산하였다.

결과: CD205, RANKL, FGFR2, EZH2, BMI1 및 C-kit 의 발현 수준은 유형 B3 흉선종 및 흉선 편평상피암종 간에 서로 유의하게 달랐다 (*P*-values of <0.001 to 0.045). 유형 B3 흉선종 및 흉선 편평상피암종을 감별함에 있어 위의 개별 마커들의 AUC 는 0.647 에서 0.878

사이였다 (*P*-values of <0.001 to 0.080). 가장 우수한 단일 마커는 EZH2 로, 민감도 (94.0 *vs*. 82.4%) 및 특이도 (80.0 *vs*. 66.7%) 측면에서 C-kit 보다 더 우수하였다. 또한, EZH2 에 C-kit 과 CD205 를 조합하면, AUC 0.992 (민감도 100%, 특이도 93.3%)로 B3 유형 흉선종과 흉선 편평상피암종의 감별 능력이 좀 더 향상되었다.

결론: EZH2, C-kit, CD205 의 조합은 B3 유형 흉선종 및 흉선 편평상피암종을 감별함에 있어 민감도와 특이도가 우수한 하나의 마커로 사용될 수 있다.

주요어: EZH2, C-kit, CD205, 흉선종, 면역조직화학염색

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